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FlhA Influences *Bacillus thuringiensis* PlcR-Regulated Gene Transcription, Protein Production, and Virulence

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***Bacillus thuringiensis* and *Bacillus cereus* are closely related. *B. thuringiensis* is well known for its entomopathogenic properties, principally due to the synthesis of plasmid-encoded crystal toxins. *B. cereus* appears to be an emerging opportunistic human pathogen. *B. thuringiensis* and *B. cereus* produce many putative virulence factors which are positively controlled by the pleiotropic transcriptional regulator PlcR. The inactivation of *plcR* decreases but does not abolish virulence, indicating that additional factors like flagella may contribute to pathogenicity. Therefore, we further analyzed a mutant (*B. thuringiensis* 407 Cry$^-$ Δ*flhA*) previously described as being defective in flagellar apparatus assembly and in motility as well as in the production of hemolysin BL and phospholipases. A large picture of secreted proteins was obtained by two-dimensional electrophoresis analysis, which revealed that flagellar proteins are not secreted and that production of several virulence-associated factors is reduced in the *flhA* mutant. Moreover, we quantified the effect of FlhA on *plcA* and *hblC* gene transcription. The results show that the *flhA* mutation results in a significant reduction of *plcA* and *hblC* transcription. These results indicate that the transcription of several PlcR-regulated virulence factors is coordinated with the flagellar apparatus. Consistently, the *flhA* mutant also shows a strong decrease in cytotoxicity towards HeLa cells and in virulence against *Galleria mellonella* larvae following oral and intrahemocoelic inoculation. The decrease in virulence may be due to both a lack of flagella and a lower production of secreted factors. Hence, FlhA appears to be an essential virulence factor with a pleiotropic role.**

Bacillus thuringiensis is a gram-positive, spore-forming species, motile by peritrichous flagella and belonging to the *Bacillus cereus* group, which also includes *Bacillus anthracis* and *Bacillus cereus* sensu stricto. *B. thuringiensis* is an entomopathogenic bacterium that produces, during the stationary phase, a large variety of Cry toxins that are active against insect larvae (43). *B. cereus* does not produce the Cry toxins and is an opportunistic human pathogen responsible for gastroenteritis (23) and local infections such as endophthalmitis (7). Although these bacteria infect distinct hosts, they share common pathogenic features. Indeed, the opportunistic properties of *B. thuringiensis* and *B. cereus* have been demonstrated in a vertebrate infection model by administering spores to mice via nasal instillation, suggesting that the two species might share common virulence factors (41).

B. thuringiensis and *B. cereus* produce many putative virulence factors that are positively controlled by the pleiotropic regulator PlcR (1, 28, 34). Expression and activation of the *plcR* regulon at the onset of the stationary phase is dependent on a quorum-sensing system involving the PapR peptide (44). About 80% of the extracellular proteins produced during stationary phase depend on PlcR (20). Among these are degradative enzymes like *p*hosphatidyl*i*nositol-preferring *p*hospholipase *C* (PI-PLC), *p*hosphatidyl*c*holine-preferring *p*hospholipase *C* (PC-PLC), hemolysins (such as the tripartite enterotoxic complex Hbl), and cytotoxins (the cytotoxin CytK) and proteases. Several studies have shown that some of these proteins might contribute (little or significantly) to virulence (3, 6–8, 14, 31). However, the precise role of these proteins in pathogenesis is not demonstrated, and it appears that none of these factors alone is sufficient to cause a virulent phenotype. Moreover, the inactivation of the *plcR* gene decreases but does not abolish the pathogenicity of *B. thuringiensis* and *B. cereus* in insects, mice, and rabbit eyes (9, 41). This suggests that additional factors, not regulated by PlcR, contribute to virulence.

Ghelardi et al. characterized a mini-Tn*10* mutant of *B. thuringiensis* that lacks flagella and is defective in its ability to swarm as well as in the secretion of both Hbl and PC-PLC proteins, although the genes were transcribed in the mutant strain (19). The transposon insertion was localized in a gene displaying similarity with *flhA*, a flagellar class II gene involved in the type III export of flagellar components in *Salmonella* (33). The FlhA flagellar basal body protein is also required in flagellum assembly and swarm cell differentiation (19, 24).

In order to further characterize this *B. thuringiensis flhA* mutant and to elucidate an eventual relationship between motility, secretion of virulence factors, and pathogenesis, we used several approaches. First, a more general picture of the secreted proteins was obtained by two-dimensional electrophoresis analysis of the extracellular proteome of the *B. thuringiensis*

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flhA mutant and the wild-type parental strains. Second, we studied whether FlhA may act at a transcriptional level, as reported for *Proteus mirabilis*, where a mutation in *flhA* resulted in modified hemolysin *hpmA* gene expression (24). Third, the toxicity of the *flhA* mutant was measured towards eukaryotic cells, and its virulence was assessed in larvae of the greater wax moth, *Galleria mellonella*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The acrystalliferous *B. thuringiensis* strain 407 Cry$^-$ (29) was used in this study. The 407 Cry$^-$ [*plcA′Z*] strain carrying a chromosomal transcriptional *plcA′-lacZ* fusion (22) and the 407 Cry$^-$ [*plcA′Z*] Δ*flhA* strain carrying an *flhA* gene inactivated by a mini-Tn*10* insertion have been described previously (19).

Escherichia coli K-12 strain TG1 [Δ(*lac-proAB*) *supE thi hsd*Δ5 (F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was used as a host for the construction of plasmids and cloning experiments. *E. coli* strain ET12567 (F′ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 glaK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1*) was used to generate unmethylated plasmid DNA prior to *B. thuringiensis* transformation. Plasmids were introduced as previously described by electroporation in both *E. coli* (13) and *B. thuringiensis* (29).

E. coli and *B. thuringiensis* were grown in Luria broth (LB) medium with vigorous shaking (175 rpm) at 37°C. The following antibiotic concentrations were used for bacterial selection: ampicillin at 100 μg ml^{-1} for *E. coli* and spectinomycin at 200 μg ml^{-1} and erythromycin at 10 μg ml^{-1} for *B. thuringiensis*. β-Galactosidase production was detected on LB plates supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 120 μg ml^{-1}.

DNA manipulations. Chromosomal DNA was extracted from *B. thuringiensis* cells as follows. Ten milliliters of exponentially growing cells was centrifuged, suspended in 400 μl of TE10 buffer (10 mM Tris HCl [pH 8], 1 mM EDTA), and treated with 5 mg lysozyme and 25 μl RNase (0,5 mg/ml) for 1 h at 37°C, and then sodium dodecyl sulfate (20%) and $NaClO_4$ (5 M) were added. Proteins were extracted by phenol treatment, and DNA was then recovered in TE10 buffer following ethanol precipitation.

Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure using Qiaprep spin columns (QIAGEN). Restriction enzymes (New England Biolabs) and T4 DNA ligase (Invitrogen) were used in accordance with the manufacturers' recommendations. Oligonucleotide primers were synthetized by Proligo (Paris, France). PCRs were performed in a PTC-100 thermocycler (MJ research, Inc.). Amplified fragments were purified using the QIAquick PCR purification kit (QIAGEN) and separated on 1% agarose gels after digestion. Digested DNA fragments were extracted from agarose electrophoresis gels using the QIAquick gel extraction kit (QIAGEN).

Plasmid constructions. The *hbl′gusA* and *plcR′gusA* fusions were constructed as follows. The *gusA* gene was extracted from pTUM177 (32) and cloned between the PstI and HindIII sites of pHT304-18 (2). The recombinant plasmid was named pHT304-18G. The 886-bp DNA fragment corresponding to the *hblC* promoter region was amplified by PCR using *B. thuringiensis* 407 chromosomal DNA as a template and primers hbl_pTUM_FW (5′-GGAATTCTTCATACT GAATATTTGTT-3′) and hbl_pTUM_RV (5′-GCTCTAGAGCCTTTACCAT TGTTTTTATAAC-3′). The 289-bp DNA fragment corresponding to the *plcR* promoter region was amplified with primers P1 (5′-GCTCTAGATTGTTAAA CCAGGCTGAG-3′) and P2 (5′-TTAACTGCAGCCCATTATAACAATCTA ATT-3′). The purified DNA fragments were digested with the appropriate restriction enzymes and then cloned between the corresponding enzyme sites of pHT304-18G. The recombinant plasmids, designated pHT304-18*hbl′G* and pHT304-18*plcR′G* were introduced into *B. thuringiensis* by electroporation.

For the *trans*-complementation of the 407 Cry$^-$ [*plcA′Z*] Δ*flhA* mutant strain with *plcA*, a 1,276-bp BamHI/PstI fragment containing the *plcA* gene with its promoter region was amplified by PCR using primers plcAFW (5′-CGCGGAT CCAGATGGTTCATACGTATTG-3′) and plcARV (5′-AAACTGCAGTACA ATTTATATTGTTGG-3′). The amplified fragment was digested with the appropriate restriction enzymes and inserted between the BamHI and PstI sites of pHT304 (2). The resulting plasmid was designated pHT304-plcA. For the *trans*-complementation of Δ*flhA* mutant strain with *flhA*, a 2,471-bp HindIII/BamHI fragment containing the *flhA* gene was amplified by PCR from the *B. cereus* ATCC 14579 chromosome using the following oligonucleotides: flh1 (5′-CCCA AGCTTGCCCGTGAACAAGAAATACC-3′) and flh4 (5′-CGCGGATCCTTC ATTCACTTCTTCCTG-3′). The amplified fragment was digested and cloned as a HindIII/BamHI DNA fragment between the HindIII and BamHI sites of pHT304. The resulting plasmid was designated pHT304-flhA.

Antibiotic and sporulation assays. LB medium containing 0.25 to 128 μg ml^{-1} ampicillin was inoculated with 407 Cry$^-$ [*plcA′Z*] or 407 Cry$^-$ [*plcA′Z*] Δ*flhA* and incubated at 37°C for 18 h. Growth was evaluated by visual observations. For sporulation assays, 407 Cry$^-$ [*plcA′Z*] and 407 Cry$^-$ [*plcA′Z*] Δ*flhA* strains were grown in HCT, a sporulation-specific medium (27), for 36 h at 30°C with vigorous shaking. The number of viable cells was counted as total CFU on LB plates. The number of spores was determined as heat-resistant (80°C for 12 min) CFU on LB plates.

β-Galactosidase and β-glucuronidase assays. Cells of *B. thuringiensis* harboring *lacZ* chromosomal or *gusA* plasmid transcriptional fusions were grown in LB medium without antibiotics at 37°C with vigorous shaking. For the determination of β-galactosidase and β-glucuronidase activity, exponentially growing cells (2 ml) were harvested and resuspended in 0.5 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 1 mM $MgSO_4$, 1 mM dithiothreitol). The cells were disrupted with glass beads (212 to 300 μm; Sigma) in a Fast-Prep 120 (Savant), and cell extract was obtained after centrifugation. Next, 0.7 ml of Z buffer and 200 μl of 2 mg ml^{-1} 2-nitrophenyl-β-D-galactoside (Sigma) for β-galactosidase assay or 200 μl of 4 mg ml^{-1} 4-nitrophenyl-β-D-glucuronide (Sigma) for β-glucuronidase assay were added to 100 μl of cell extract. The mixture was incubated at 37°C, and the reaction was stopped by the addition of 0.2 ml of 2 mM Na_2CO_3. Subsequently, the optical density of the reaction mixture was measured at 420 nm or 405 nm for β-galactosidase or β-glucuronidase assay, respectively. The protein content was determined using the Bio-Rad protein assay with bovine serum albumin as the standard. Specific activities are expressed in units of β-galactosidase and β-glucuronidase per milligram of protein (Miller units).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was done as described previously (20). Briefly, the culture supernatant of 407 Cry$^-$ and 407 Cry$^-$ [*plcA′Z*] Δ*flhA* was collected 2 h after the onset of stationary phase, centrifuged at 8,000 rpm, and filtered. Proteins were precipitated using the deoxycholic acid-trichloroacetic acid method (37). The pellet was washed with ethanol ether (1:1) and dissolved in a urea-thiourea-CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}-ampholine mixture. A total of 20 μg of proteins was loaded onto each immobilin polyacrylamide gel strip (17-cm length, in the linear pH range of 4 to 7) for the first dimension, and electrofocusing was performed on an Amersham Pharmacia Multiphor II horizontal electrophoresis system for a total of 35,000 Vh. The strips were then equilibrated first in urea-sodium dodecyl sulfate-Tris-dithiothreitol and then in urea-sodium dodecyl sulfate-Tris-acetamide. The second dimension was performed on a 10 to 12.5% gradient acrylamide gel. Gels were silver stained (38) and scanned at 300 dpi and at 8-bit depth on a SHARP JX-330 scanner equipped with a film scanning unit. Protein identification was determined by comparison to a reference gel (21) or by mass spectrometry.

Cell cultures and cytotoxicity assays. Epithelial HeLa cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells were incubated at 37°C under a 5% CO_2 atmosphere and saturating humidity. Cells were detached using 0.02% trypsin, counted with a hematocytometer, and seeded into multiwell disposable trays containing DMEM plus fetal bovine serum at a density of 2×10^5 cells per well for 24 h. The culture supernatants of 407 Cry$^-$ [*plcA′Z*] and 407 Cry$^-$ [*plcA′Z*] Δ*flhA*, grown in LB medium at 37°C under agitation until early stationary phase, were collected, centrifuged at 4,000 rpm, and filtered using a 0.2-μm filter. New DMEM medium was added, and cells were infected with the culture supernatants (final dilution, 1/25). After 2 h, trypan blue dye was added to the preparation. Nonpermeabilized cells remained unstained, whereas permeabilized cells allowed the dye to enter inside the cytoplasm, and cells were therefore stained blue. At least 300 cells were visually counted, and the percentage of blue cells compared with unstained cells accounted for the percentage of cytotoxicity. Results are mean values of three independent experiments.

Insects and in vivo experiments. *Galleria mellonella* eggs were hatched at 25°C, and the larvae were reared on bee's wax and pollen (Naturalim). Trypsin-activated Cry1C toxin was prepared from the asporogenic *B. thuringiensis* strain 407 Δ*sigK* (5) transformed with pHT1C (42). Crystals were purified on a 67 to 72% sucrose gradient and solubilized in 0.1 M $NaCO_3$ carbonate buffer (pH 10.3), dialyzed against 0.1 M sodium phosphate buffer (NaPi) (pH 8.5), and activated by incubation with trypsin (2% [wt/wt] protein) for 3 h at 37°C.

For the infection experiments, groups of 20 to 30 last-instar *G. mellonella* larvae, weighing around 200 mg, were force-fed with 10 μl of a mixture containing 5×10^6 vegetative bacteria (grown in LB medium at 37°C until an optical density at 600 nm of 1 to 2 was reached) and 2 μg purified and activated Cry1C toxin (10 μl/larva) or with 10 μl toxin or bacteria alone using a 0.5- by 25-mm

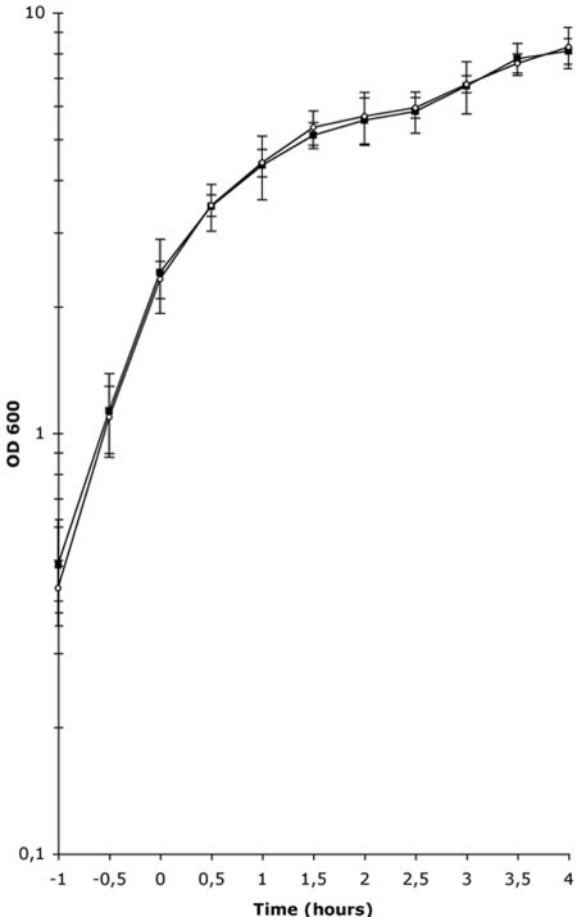

FIG. 1. Growth curves of *B. thuringiensis* 407 Cry$^-$ [*plcA′Z*] (■) and *B. thuringiensis* 407 Cry$^-$[*plcA′Z*] Δ*flhA* (○) in LB medium at 37°C. Each point is the mean of five independent experiments. Vertical bars indicate standard errors. OD 600, optical density at 600 nm.

needle and a microinjector (Burkard Manufacturing). The larvae were kept in individual boxes at 37°C. A control group was fed with NaPi buffer. Mortality was recorded after 24 h. Infection by injection into the hemolymph was performed as follows. Groups of 25 larvae were injected at the base of last proleg with 10 μl vegetative bacterium suspension using the Burkard microinjector with a 1-ml syringe and 0.45- by 12-mm needles (Terumo). To estimate *B. thuringiensis* cells in alive or dead larvae, 10 insects were crushed and homogenized in 10 ml sterile water; dilutions were plated onto LB agar plates containing appropriate antibiotics: oxacillin (10 μg ml^{-1}) for the parental strain and spectinomycin (200 μg ml^{-1}) for the mutant strain. To estimate whether just-dying larvae (17 h after oral infection) contained vegetative bacteria or spores, samples of crushed and homogenized larvae were submitted to a heat treatment (70°C for 15 min) before dilution and plating. All tests were run at least three times.

Statistical analysis. The mortality data following vegetative cell injections were analyzed by calculating 50% lethal doses using the Log-Probit program (18, 39).

RESULTS

Phenotypical features of the mutant strain. We further analyzed the *B. thuringiensis* 407 Cry$^-$ [*plcA′Z*] Δ*flhA* mutant previously described as negatively affected in motility and in hemolytic activity on sheep blood plates (19). First, we showed that the Δ*flhA* mutation did not affect the kinetics of growth in LB medium at 37°C (Fig. 1). Second, we assessed the sporulation frequencies. Plating of cultures grown for 36 h at 30°C in HCT medium following heat treatment showed that a large proportion (89%) of the parental 407$^-$ [*plcA′Z*] gave heat-resistant spores. The Δ*flhA* mutation conferred a sporulation-defective phenotype (<0.003% sporulation). These results suggested that FlhA plays an important role in the triggering or in the development of the sporulation process. Indeed, intracellular condensation and prespore formation were observed.

It was previously reported that in an avirulent mutant of *B. thuringiensis*, the expression of flagellin, phospholipase C, and β-lactamase was concomitantly abolished (46). Thus, we tested the *flhA* mutant for ampicillin resistance. The MIC of ampicillin for the *flhA* mutant was determined in LB medium. The 407$^-$ [*plcA′Z*] was able to grow with up to 16 μg ml^{-1} ampicillin, while the Δ*flhA* mutant strain was sensitive to 0.5 μg ml^{-1} ampicillin, indicating a clear reduction of resistance to ampicillin.

Effect of the *flhA* mutation on extracellular protein production. The secretion of virulence-associated proteins such as Hbl and PC-PLC depended on the presence of *flhA* (19). We therefore investigated the possible implication of the *flhA* gene on the production of a larger number of extracellular factors by two-dimensional electrophoresis. The comparison of the extracellular proteomes revealed several differences between the *flhA* mutant and the parental strain. Although the growth curves were similar, the protein concentration in the culture supernatant at T_2 (T_n indicates the number of hours from the onset of the stationary phase), determined using the Bradford method (4), was twofold higher for the wild-type strain (186 ± 16 μg and 87 ± 15 μg for the wild-type and the mutant strains, respectively). This suggested a generally lower protein production for the mutant.

Furthermore, among major differences between the culture supernatants at T_2 from the mutant strain and those from the wild type (Fig. 2A and B), the lack of flagellin, the lack of secretion of Hbl components L1 and L2, and a decrease of metalloprotease InhA2 were observed. Additionally, although a degraded form of Hbl component B was detected at around 10 kDa, the mature form was not identified. This observation might suggest that Hbl component B was degraded and that Hbl components L1 and L2 were not secreted by the *flhA* mutant strain (Fig. 2B). In comparison with gel diffusion assays performed previously by Ghelardi et al., which indicated that *flhA* was also involved in PC-PLC production, our two-dimensional gel revealed the presence of several forms of PC-PLC. This indicates that the mutation in *flhA* does not affect PC-PLC secretion. Since the *flhA* mutant is obtained in a strain carrying a *plcA* gene knockout (by a chromosomal transcriptional fusion between the *plcA* gene promoter and the *lacZ* gene), no PI-PLC was found in the secretome. Introduction of the pHT304-plcA plasmid into the Δ*flhA* strain restored PI-PLC secretion (data not shown). These results confirm that *flhA* is required for the production of flagellins, of the major Hbl components, and of other PlcR-regulated factors like InhA2.

Effect of the *flhA*-null mutation on *plcR*, *hblC*, and *plcA* gene transcription. The differences observed in the secretome of the wild-type and *flhA* mutant strains might both result from a reduction in secretion and in transcription. We found that the *flhA* mutant strain, carrying a chromosomal *plcA′-lacZ* transcriptional fusion, gave light blue colonies on LB plates containing X-Gal, indicating that expression of the *lacZ* gene was lower in the *flhA* mutant strain than in the parental strain. We

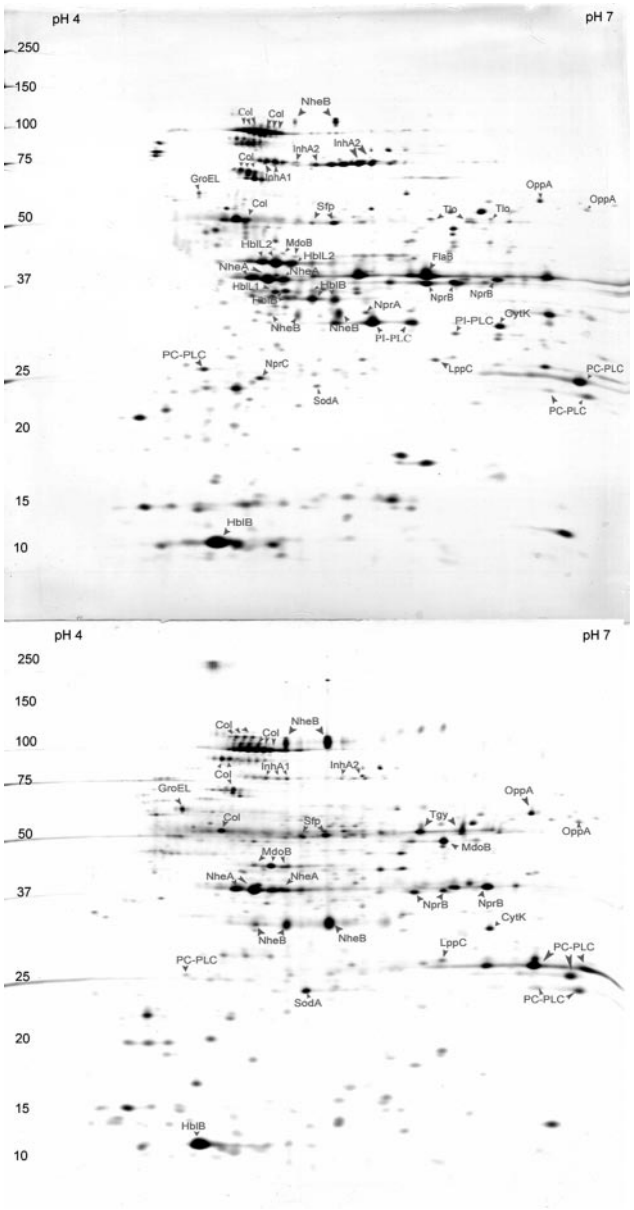

FIG. 2. Comparison of two-dimensional gels from *B. thuringiensis* 407 [*plcA′Z*] (A) and *B. thuringiensis* 407 [*plcA′Z*] Δ*flhA* (B) strains. For each strain, proteins were extracted from the culture supernatant harvested at T_2 in stationary phase. Twenty micrograms of these proteins was loaded onto immobilin polyacrylamide gel strips in the linear pH range of 4 to 7 and were separated by two-dimensional electrophoresis and silver stained. Protein identification was determined by mass spectrometry or by comparison with previously published reference gels.

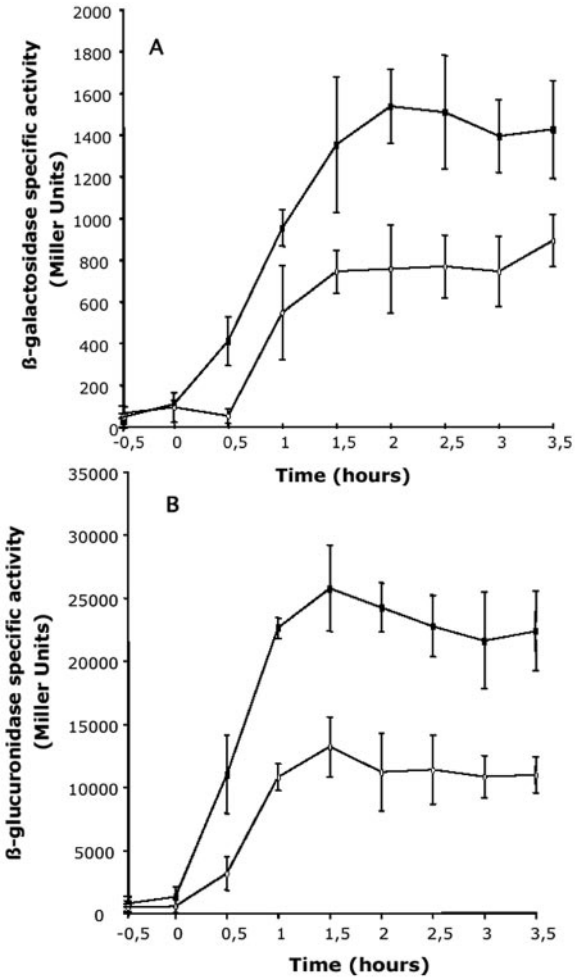

FIG. 3. Effect of *flhA* mutation on *plcA* (A) and *hblC* (B) transcriptions. (A) β-Galactosidase activity of the *B. thuringiensis* 407 Cry⁻ [*plcA′Z*] (■) and *B. thuringiensis* 407 Cry⁻ [*plcA′Z*] Δ*flhA* (○) strains. (B) β-Glucuronidase activity of the *B. thuringiensis* 407 Cry⁻ [*plcA′Z*] (■) and *B. thuringiensis* 407 Cry⁻ [*plcA′Z*] Δ*flhA* (○) strains carrying pHT304-*hbl′G*. The cells were grown at 37°C in LB medium. Time zero indicates the onset of the stationary phase, and T_n is the number of hours before (−) or after time zero. Each point is the mean of three or four independent experiments. Vertical bars indicate standard errors.

measured the effect of the *flhA*-null mutation on the kinetics of *plcA* expression. β-Galactosidase activity in bacteria growing in LB medium at 37°C was investigated (Fig. 3A). Both parental 407 Cry⁻ [*plcA′Z*] and mutant 407 Cry⁻ [*plcA′Z*] Δ*flhA* cells started to express β-galactosidase at T_0 and $T_{1/2}$, respectively. The maximum activity was reached 1 h 30 min after the onset of the expression; however, β-galactosidase activity was twofold reduced in the Δ*flhA* mutant strain. It was previously described that in *Proteus mirabilis*, expression of *hpmA* (encoding a hemolysin) and *flhA* was coordinated (24). To determine whether the *flhA* mutation also influenced the expression of *hbl* in *B. thuringiensis*, a plasmid carrying a transcriptional fusion between the *hblCDAB* promoter *hblp* and the *gusA* gene was introduced into the 407 Cry⁻ [*plcA′Z*] and 407 Cry⁻ [*plcA′Z*] Δ*flhA* strains. Bacteria were grown in LB medium at 37°C, and the β-glucuronidase activity was measured. No difference in growth curves was found between the 407 Cry⁻ [*plcA′Z*] and the 407 Cry⁻ [*plcA′Z*] Δ*flhA* strains harboring the plasmid pHT304-18*hbl′G* (data not shown). β-Glucuronidase activity in the *flhA* background was about 50% of that displayed by the parental strain (Fig. 3B). These results suggested that the *flhA* gene is involved in both *plcA* and *hbl* gene transcription. Since *plcA* and *hbl* belong to the PlcR regulon (1), we investigated

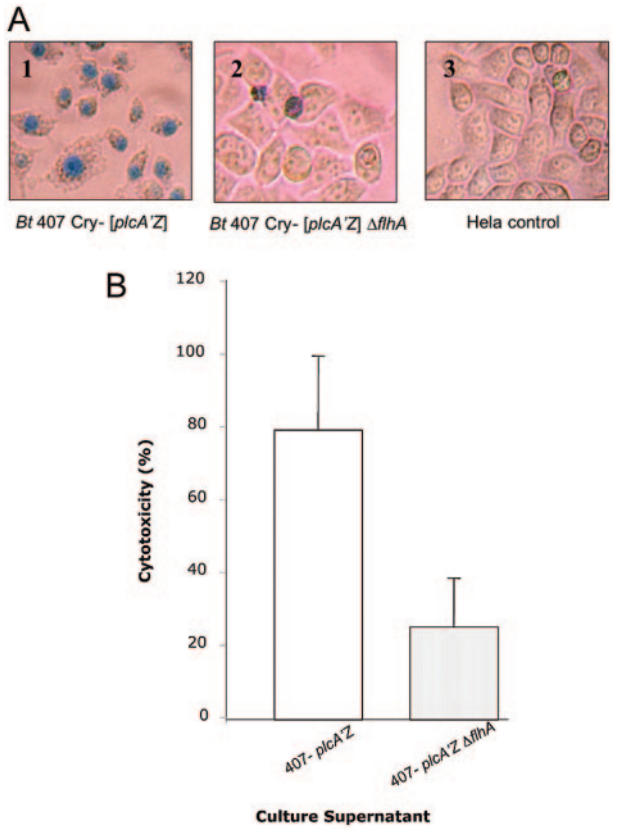

FIG. 4. Cytotoxicity to HeLa cells is FlhA dependent. (A) Cells were infected with the supernatant of *B. thuringiensis* 407 Cry$^-$ [*plcA′Z*] (1) and *B. thuringiensis* 407 Cry$^-$ [*plcA′Z*] Δ*flhA* (2) strains at a final dilution of 1:25. After 2 h, trypan blue dye was added to the cells. Untreated control cells are also shown (3). Nonpermeabilized cells remained unstained, whereas permeabilized cells allowed the dye to enter inside the cytoplasm, and cells were therefore stained blue. (B) At least 300 cells were visually counted, and the percentage of blue cells compared with that of unstained cells accounted for the percent cytotoxicity. Results are mean values of three independent experiments.

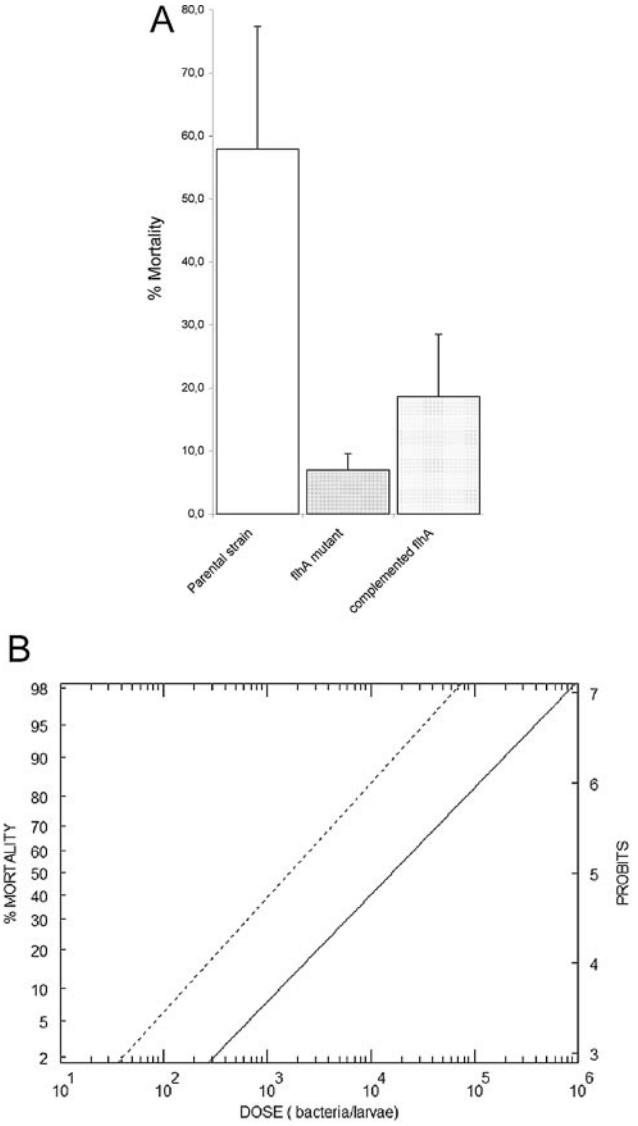

FIG. 5. Effect of *flhA* mutation on virulence against the insect *G. mellonella* by force-feeding (A) and intrahemocoelic injection (B). (A) Last-instar larvae were force-fed with 2 μg Cry1C toxin and 5×10^6 log-phase bacteria larva^{-1}. No mortality was observed with Cry1C toxin or vegetative bacteria alone. Results are mean values of four independent experiments. Verticals bars indicate the standard errors of the means. (B) Dose-mortality responses observed after injection into the hemocoel of *B. thuringiensis* 407 Cry$^-$ [*plcA′Z*] (hatched line) and *B. thuringiensis* 407 Cry$^-$ [*plcA′Z*] Δ*flhA* (solid line) vegetative bacteria. No mortality was observed in the control (NaPi buffer alone).

whether *flhA* directly controlled *plcR* expression by measuring the expression of a transcriptional fusion between the *plcR* promoter region *plcRp* and the *gusA* gene. The plasmid pHT304-18*plcR′G* carrying this transcriptional fusion was introduced into the 407 Cry$^-$ [*plcA′Z*] and *flhA* mutant strains. We assessed the kinetics of β-glucuronidase activity in bacteria grown in LB medium. The β-glucuronidase activity was low in the two strains (ranging from 50 to 100 Miller units), and no significant difference was observed between the two strains (results not shown). These results indicate that a functional FlhA is required for the full expression of at least two PlcR-regulated genes (*hbl* and *plcA*). However, it appears that this effect is independent of PlcR.

Cytotoxicity. *B. thuringiensis* is cytotoxic towards, for instance, insect hemocytes, and some of the cytotoxic components, secreted during bacterial growth, are PlcR regulated (20, 41). Cytotoxicity to epithelial cells presumably occurs through the destruction of epithelial cells by bacterial secreted factors (pore-forming toxins, enzymes, etc.). The results described above suggest that FlhA controls the expression of several virulence-associated genes. Thus, we investigated the activity of culture supernatants from the 407 Cry$^-$ parental strain and from the 407 Cry$^-$ Δ*flhA* mutant strain against HeLa cells. A trypan blue test was used to determine the role of the *flhA* mutation in cell membrane alterations. The supernatant of the parental 407 Cry$^-$ [*plcA′Z*] strain was cytolytic: the HeLa cells lost their shape and were stained with trypan blue, indicating cell death (Fig. 4A). By comparison, most of the cells were not killed by the supernatant of the 407 Cry$^-$ Δ*flhA* mutant strain. The supernatant of parental strain 407 Cry$^-$ [*plcA′Z*] induced a strong cytotoxicity, and after 2 h of

infection, 80% of the cells had permeabilized membranes (Fig. 4B). In contrast, the *flhA* mutant strain had a reduced cytotoxic capacity, and after 2 h, only 25% of the cells were affected. Thus, FlhA may play a role in factors involved in host cell membrane permeabilization, and this could contribute to virulence.

Pathogenicity in insects. To assess the pathogenicity of *B. thuringiensis* against insects, vegetative bacteria were either fed to the larvae in association with the insecticidal crystal toxin Cry1C or injected alone into the hemolymph. The lepidopteran *G. mellonella* larva is not or is only weakly susceptible to the ingestion of *B. thuringiensis* vegetative bacteria, spores, or Cry toxins alone. However, bacteria and Cry toxins sometimes act in synergy, and a stronger mortality is obtained by mixing vegetative bacteria or spores and Cry toxins; that is the reason why *G. mellonella* has proven to be useful for identifying *B. thuringiensis* virulence factors that are different from Cry toxins (16, 30, 41). Indeed, less than 2% mortality was observed with Cry1C toxin (2 μg larva^{-1}), and no mortality was observed with vegetative bacteria alone (5×10^6 bacteria larva^{-1}) (results not shown). The synergism between activated toxin and vegetative bacteria from the Δ*flhA* mutant and the *flhA* complemented strain was compared to the effect of the parental 407 Cry$^-$ [*plcA′Z*] strain (Fig. 5A). Strong mortality was obtained (58% mortality) with the parental 407 Cry$^-$ [*plcA′Z*] strain, and virulence was significantly reduced with the Δ*flhA* mutant (6.8% mortality), while the virulence was partially restored with the complemented strain (18.7% mortality).

The pathogenesis of the 407 Cry$^-$ [*plcA′Z*] and the 407 Cry$^-$ [*plcA′Z*] Δ*flhA* mutant strains was also assessed by injecting the vegetative bacteria into the larval hemolymph (Fig. 5B). The mortality curves were significantly different. The 50% lethal doses (with the confidence intervals in parentheses), determined 24 h after the injection of log-phase bacteria, were 1,646 (815 to 3,262) CFU for the parental strain and 15,923 (4,491 to 68,003) CFU for the mutant strain. Although the growth curves were not significantly different in LB medium at 37°C, we assessed the multiplication of the two strains in larva by bacterial counts 24 h postinjection of 10^4 bacteria/larva. This dose resulted in 85% and 40% mortality of the larvae infected with the parental and the mutant strains, respectively. No difference in bacterial counts was observed either in living larva (3.1×10^5 CFU larva^{-1} and 2.9×10^5 CFU larva^{-1} for the parental and the mutant strains, respectively) or in dead ones (10^8 CFU per caterpillar for both strains). Thus, the difference in virulence observed for the *flhA* mutant was probably not due to the lack of multiplication or to a slower bacterial growth rate. Since the *flhA* mutant was found to be inefficient in sporulation, we also tested whether this feature could explain the decrease in virulence. This was not found; all dead larvae (17 h after ingestion of 5×10^6 bacteria and 2 μg Cry1 toxin) were killed before any bacteria had completed sporulation. In fact, 2×10^8 CFU per larva were found before heat treatment, and none resisted 70°C for 15 min. These results indicated that FlhA plays a crucial role during the infectious process of *G. mellonella* both by oral and by intrahemocoel routes.

DISCUSSION

Ghelardi et al. indicated that FlhA was required for flagellin export and secretion of virulence-associated proteins such as hemolysin BL and phosphatidylcholine-preferring phospholipase C (19). However, no evidence was provided to state whether FlhA affects the production of virulence factors at a transcriptional, posttranscriptional, or posttranslational level. We thus investigated the effect of the *flhA* mutation on general extracellular protein production 2 h after the onset of the stationary phase (corresponding to the major expression of the PlcR regulon) (20) by two-dimensional electrophoresis. As expected, in the *B. thuringiensis flhA* mutant, we observed the lack of flagellins and Hbl L1 and L2 products. However, we found a degraded form of Hbl component B, and the secretion of PC-PLC was not reduced by the *flhA* mutation (Fig. 2). The two-dimensional electrophoresis also reveals a striking reduction of the metalloprotease InhA2 in the extracellular proteome of the *flhA* mutant. The generally lower (twofold) amount of secreted proteins found in the culture supernatant of the *flhA* mutant might be partly due to the lack of flagellins which are among the most abundant proteins found in the extracellular proteome of the parental strain. However, this general reduction of extracellular proteins is more likely due to a lower transcription of some PlcR-regulated genes like *hblC* and *plcA*. The mechanism that negatively controls these genes in the *flhA*-deficient strain remains unknown.

The absence of the Hbl L1 and Hbl L2 components in the Δ*flhA* background suggests that they were not secreted by the mutant. However, the degraded form of HblB found in the extracellular proteome may be due to a higher proteolytic activity in the Δ*flhA* background. Silver staining of the two-dimensional electrophoresis gel revealed weak spots around the presumed localization of L1 and L2, but the these spots were too weak to be identified by matrix-assisted laser desorption ionization–time of flight. Thus, we cannot exclude that traces of the other Hbl components were present on the mutant strain gel. Altogether, these results suggest that the absence of Hbl components in the culture supernatant of the *flhA* mutant might be due to a decrease in *hbl* operon transcription and to a low stability of these proteins, rather than a secretion defect.

As part of the pleiotropic phenotype, the *flhA* mutant also showed an ampicillin-sensitive phenotype. Similarly, a flagella mutant of *B. thuringiensis* isolated by Heierson et al. (25) was found to produce fewer (twofold) β-lactamases than the wild-type strain. Moreover, It has been previously described that in *B. anthracis*, low expression of *bla1* and *bla2* genes (encoding functional β-lactamases) is not sufficient to confer resistance to β-lactam agents (12). Thus, the ampicillin-sensitive phenotype of the *flhA* mutant might result from a decrease in transcription of β-lactamase genes.

Furthermore, the Δ*flhA* mutation also conferred a sporulation-defective phenotype. In *B. subtilis*, spore formation is initiated by integrating a wide range of environmental and physiological signals (i.e., nutrient depletion and cell density) that, when channeled into a phosphorelay, activate a key transcriptional regulatory protein, Spo0A. At least three protein kinases transfer phosphate from Spo0F to Spo0A (26). In addition, the phosphorylation state of Spo0A is modulated by a specific phosphatase (35, 36). In *B. subtilis*, FlhA is a possible candidate for a membrane-bound signaling molecule implicated in gene expression (11). Thus, FlhA might be involved in the expression and the stability of a molecule that is required for the

development of sporulation. A prerequisite event for infection is the contact of pathogenic bacteria with the target tissue. Epithelial cells represent the first and the major cell type encountered by microorganisms in mucosa and therefore constitute the main sites of host-pathogen interactions. Here, we show that *B. thuringiensis* is highly cytotoxic to epithelial cells and that this cytotoxic activity depends on FlhA. The low cytotoxicity of the *flhA* mutant is likely due to the reduced production of various extracellular factors. A mutant lacking *flhA* is less cytotoxic and might therefore be impaired in its capacity to penetrate through deeper tissues and to colonize its host.

Flagella have been shown to play an important role in the virulence of many bacterial pathogens, including *Salmonella*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* (17, 40, 45), due to their role in mobility, adhesion, and induction of immunoresponses. The effect of the *flhA* mutation on the pathogenicity of *B. thuringiensis* was assessed against *G. mellonella* larvae, which is an ideal insect "model" to measure the effect of chromosomal virulence factors of *B. thuringiensis* or *B. cereus* (16, 41), since it is only weakly susceptible to Cry toxin. In this study, virulence was strongly decreased by both force-feeding and intrahemocoelic injection. This is the first demonstration of the role of FlhA in the virulence of *B. thuringiensis* in insects, but a recent study showed an effect on rabbit endophthalmitis (10). Moreover, so far, no other *B. thuringiensis* factors have been described to play a role by both oral and intrahemocoel inoculation. Previously, it was demonstrated that the PlcR-regulated factors are more important for virulence against *G. mellonella* via the oral route rather than by injection into the hemolymph (41). The role of mobility might be minor, since no change in virulence was recorded upon injection of a nonmotile mutant, *B. thuringiensis* 407 Cry$^-$ Δ*clpP2*, into the hemolymph of *Bombyx mori* larvae (15). Our results also show that *B. thuringiensis* vegetative bacteria are able to kill *G. mellonella*; sporulation is not necessary to achieve larval mortality. Although we could not demonstrate a direct role for the *flhA* mutation in *plcR* gene transcription, we have shown that the production of several factors dependent on PlcR was reduced in the *flhA* mutant. This is notable in the case of the metalloprotease InhA2, which is important for virulence of *B. thuringiensis* against *G. mellonella* larvae (16). Our study gives further insight into the pleiotropic effect of FlhA and indeed shows the importance of FlhA for virulence. It also indicates that the phospholipases PI-PLC and PC-PLC may not be major virulence factors, since PI-PLC is already absent from the virulent parental strain and PC-PLC is present in both the parental strain and the mutant. This is new, since Ghelardi et al. (19) indicated the absence of PC-PLC in the *flhA* mutant. A minor role for these phospholipases in endophthalmitis was reported previously by Callegan et al. (7) as well. Our results may also suggest that the unknown mutation in the avirulent pleiotropic *B. thuringiensis* mutant, described previously by Zhang et al. (46), could be a mutation in *flhA*. Meanwhile, the pleiotropic phenotype of the *flhA* mutant does not allow differentiation of the individual role of the absence of flagella, the reduced motility, or the decrease in production of extracellular components in virulence. Therefore, the precise determination of the roles of each phenotype in cytotoxicity and virulence requires additional studies.

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